Food Control 62 (2016) 257–263

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Effect of tea tree oil on *Staphylococcus aureus* growth and enterotoxin production

Ce Shi [a], Xingchen Zhao [a], Haiyang Yan [a], Rizeng Meng [b], Yan Zhang [a], Wenli Li [a], Zonghui Liu [a], Na Guo [a, *]

[a] Department of Food Quality and Safety, College of Food Science and Engineering, Jilin University, 130062 Changchun, PR China
[b] Jilin Enrty-exit Inspection and Quarantine Bureau, 130062 Changchun, PR China

ARTICLE INFO

Article history:
Received 29 July 2015
Received in revised form
29 October 2015
Accepted 31 October 2015
Available online 3 November 2015

Keywords:
Staphylococcus aureus
Tea tree oil
Inhibition
Milk
Staphylococcal enterotoxin

Chemical compounds studied in this article:
(+)-Terpinen-4-ol (PubChem CID: 2724161)
α-Terpineol (PubChem CID: 17100)
α-Pinene (PubChem CID: 6654)
γ-Terpinene (PubChem CID: 7461)
1,8-Cineole (PubChem CID: 2758)

ABSTRACT

Staphylococcus aureus (*S. aureus*), a major food-borne pathogen, causes disease in mammalian hosts by producing a wide variety of exoproteins, such as α-hemolysin and staphylococcal enterotoxins. Tea tree oil (TTO), an essential oil, has broad-spectrum antimicrobial activity. The objectives of this study were to evaluate the inhibitory effects of TTO on *S. aureus* growth and on α-hemolysin, enterotoxins A and B production. In this study, the effect of TTO on *S. aureus* growth in laboratory medium and pasteurized milk was determined by time-kill assays. Treatment with half of minimal inhibitory concentration (MIC) of TTO demonstrated very little or no reduction in numbers of viable ATCC 29213 cells; however, 1 × MIC of TTO reduced the viable cell count more noticeably, and 2 × MIC of TTO demonstrated an even greater reduction in the viable cell count, both in TSB and milk. The influence of TTO on enterotoxins was determined by real-time reverse transcriptase-PCR (real-time RT-PCR), a hemolysis assay, Western blot and a tumor necrosis factor alpha (TNF-α) assay. The real-time RT-PCR results revealed that the transcription of genes encoding α-hemolysin, staphylococcal enterotoxin A (SEA) and staphylococcal enterotoxin B (SEB) were down regulated after *S. aureus* was exposed to TTO (0.0625 mg/mL-0.5 mg/mL). The hemolytic assay showed that *S. aureus* hemolytic activity was inhibited by subinhibitory concentrations of TTO (0.0625 mg/mL-0.5 mg/mL). The Western blot assay identified that the production of the three virulence factors was inhibited by TTO (0.0625 mg/mL-0.5 mg/mL). Moreover, the enzyme linked immunosorbent assay (ELISA) demonstrated that TNF-α production was suppressed by TTO treatment in RAW264.7 cells stimulated by *S. aureus* supernatant containing staphylococcal enterotoxins (SEs).

1. Introduction

Microbial contamination causes food spoilage and food-borne diseases. *Staphylococcus aureus* is a major human pathogen that causes a wide range of diseases, including food poisoning (Tranter, 1990; Le Loir, Baron, & Gautier, 2003; Pereira et al., 2009). The continuing emergence of food poisoning caused by *S. aureus* strains threatens public health and safety. Furthermore, milk and dairy products are often implicated in staphylococcal outbreaks and serve as an ideal medium for the growth of various microorganisms (De Buyser, Dufour, Maire, & Lafarge, 2001; Delbes, Alomar, Chougui, Martin, & Montel, 2006). The occurrence of *S. aureus* toxicity largely depends on the extracellular virulence factors produced by the strain, such as α-hemolysin, SEs, toxic shock syndrome toxin-1 and other factors (Dinges, Orwin, & Schlievert, 2000), which are produced throughout the logarithmic phase of growth and during the transition from the exponential to the stationary phase in a growth-phase-dependent manner (Novick, Christie, & Penadés, 2010; Koszczol, Bernardo, Kronke, & Krut, 2010). The SEs comprise a group of heat stable and serologically diverse proteins. Twenty different types of SEs, SEA-SEE, SEG-SER and SEU have already been discovered; however, SEA-SEEs are the most common enterotoxins involved in staphylococcal food poisoning (Jørgensen et al., 2005), such as SEA and SEB, and they are etiologic agents (Principato & Qian, 2014). SEs secreted by *S. aureus* stimulate cells of the immune system, such as macrophages, resulting in the release of TNF and other proinflammatory cytokines (Balaban & Rasooly, 2000; Bernardo et al., 2004; Dinges et al., 2000). The 33-kDa α-hemolysin, one of the major

* Corresponding author.
E-mail address: jlnaguo@126.com (N. Guo).

http://dx.doi.org/10.1016/j.foodcont.2015.10.049
0956-7135/

extracellular virulence factors of *S. aureus*, is a pore-forming protein that has hemolytic, dermonecrotic and cytolytic activities (Qiu, Feng, & Xiang et al., 2010; Qiu, Wang, & Xiang et al., 2010).

Based on these serious phenomena, the development of strategies to effectively suppress the growth and exoprotein production of *S. aureus* in foods, thereby improving shelf-life and food quality, is of great interest (de Souza, de Barros, da Conceição, Neto, & da Costa, 2009; Barros et al., 2009). In recent years, interest has grown in natural medicinal products, essential oils and other botanicals because of their effective antimicrobial properties against a broad spectrum of food-related pathogenic microorganisms (Smith-Palmer, Stewart, & Fyfe, 2004; Bajpai, Rahmana, Choic, Yound, & Kanga, 2007). Plants have been a valuable source of natural products for maintaining human health (Ben Hsouna, Hamdi, Miladi, & Abdelkafi, 2014). TTO, an essential oil distilled from *Melaleuca alternifolia*, is composed of terpene hydrocarbons, mainly monoterpenes, sesquiterpenes, and their associated alcohols (Raman, Weir, & Bloomfield, 1995; Banes-Marshall, Cawley, & Phillips, 2001). This compound has gained the attention of scientists, physicians and consumers due to its broad-spectrum antimicrobial and anti-inflammatory activities against a variety of bacteria, including *S. aureus* (Brady, Loughlin, Gilpin, Kearney, & Tunney, 2006; Kwieciński, Eick, & Wójcik, 2009). Moreover, drugs and care products containing TTO are frequently used to treat skin, oral, vaginal and airway infections or as antiseptics and disinfectants (Buckle, 2003; Carson, Hammer, & Riley, 2006; Kwieciński et al., 2009). Although previous researchers have described the antimicrobial activities of TTO against food-related microorganisms (Carson et al., 2006, 1995), little is known about the antimicrobial mechanism of TTO on microbial pathogenicity. Moreover, the effects of TTO on virulence-associated proteins produced by *S. aureus* remain uncharacterized (Park, Jang, Cho, & Choi, 2007). Therefore, the continuing development of antimicrobial compounds to inhibit the growth of and production of virulence factors by *S. aureus* is an active area of research. In this study, our objective was to investigate the influence of subinhibitory concentrations of TTO on *S. aureus* growth in pasteurized milk and on the production of several key virulence-associated factors, α-hemolysin and two major enterotoxins (SEA and SEB).

2. Materials and methods

2.1. Chemical reagents

TTO was obtained from Nanjing Chemlin Chemical Industry Co., Ltd. (Nanjing, China). The Mueller-Hinton (MH) broth used to test antimicrobial susceptibility, the Tryptic Soy Broth (TSB) and the Baird-Parker agar were purchased from Qingdao Hope Bio-Technology Co., Ltd. (Qingdao, China). Dulbecco's modified Eagle's medium (DMEM), obtained from Corning (USA), and fetal bovine serum (FBS), obtained from Invitrogen-Gibco (USA), were used for the cell culture. The pasteurized milk was purchased from the local supermarket.

2.2. Bacterial strains, cells and culture conditions

In this study, eighteen food-borne *S. aureus* isolates, obtained from the Jilin Entry-Exit Inspection and Quarantine Bureau, and *S. aureus* strain ATCC 29213, obtained from the China Medical Culture Collection Center, were used for quality control (Table 2). The RAW264.7 macrophage cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China, and the cells were cultured in DMEM with 10% FBS.

Table 1
Primers used for real-time RT-PCR in the study.

Genes	Primer	Sequence
agrA	*agrA*-fw	5′-TGATAATCCTTATGAGGTGCTT-3′
	agr-rv	5′-CACTGTGACTCGTAACGAAAA-3′
16S rRNA	*16S rRNA*-fw	5′-GCTGCCCTTTGTATTGTC-3′
	16S rRNA-rv	5′-AGATGTTGGGTTAAGTCCC-3′
hla	*hla*-fw	5′-TTGGTGCAAATGTTTC-3′
	hla-rv	5′-TCACTTTCCAGCCTACT-3′
sea	*sea*-fw	5′-ATGGTGCTTATTATGGTTATC-3′
	sea-rv	5′-CGTTTCCAAAGGTACTGTATT-3′
seb	*seb*-fw	5′-TGTTCGGGTATTTGAAGATGG-3′
	seb-rv	5′-CGTTTCATAAGGCGAGTTGTT-3′
sarA	*sarA*-fw	5′-TCTTGTTAATGCACAACAACGTAA-3′
	sarA-rv	5′-TGTTTGCTTCAGTGATTCGTTT-3′

2.3. Antimicrobial susceptibility testing

To determine the MICs of TTO for the above-mentioned *S. aureus* strains (Table 2), antimicrobial susceptibility testing was performed according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2009) and Zhao, Liu, Li, and et al (2014). In brief, overnight cultures (37 °C, shaking) of *S. aureus* strains in TSB were diluted with MH broth to a final concentration of 10^5 colony-forming units (CFU)/mL. TTO was prepared in MH broth to obtain subinhibitory concentrations by serial dilutions in a 96-well plate. A well without TTO served as a positive growth control. In total, 50 μL of the bacterial suspension and 50 μL of TTO dilutions of different subinhibitory concentrations were added into individual wells of a 96-well microtiter plate. Then, the plate was incubated at 37 °C for 24 h. The MICs were defined as the lowest concentration of antibiotic that produced complete inhibition of visible growth. To verify experimental accuracy, all tests were performed in triplicate.

2.4. Time-kill assay

The bactericidal activity of TTO against *S. aureus* ATCC 29213 was evaluated by measuring the reduction in the numbers of CFU; the time-kill assay was performed using a method based on previous studies (Carson, Mee, & Riley, 2002; Brady et al., 2006; Ananda, Kazmer, Hinckley, Andrew, & Venkitanarayanan, 2009). The bactericidal kinetics of TTO were studied by inoculating pasteurized milk and TSB containing 1/2 × MIC, 1 × MIC and 2 × MIC of TTO with an initial inoculum of 1×10^5 CFU/mL. Briefly, the samples

Table 2
TTO against *S. aureus* food-borne isolates and *S. aureus* ATCC 29213 in vitro.

Strains	MIC (range) of compoud (mg/mL)
S. aureus JL-20110	1 (1)
S. aureus JL-20111	1 (1)
S. aureus JL-20112	1 (1)
S. aureus JL-20113	2 (1–2)
S. aureus JL-20114	1 (1)
S. aureus JL-20115	1 (1)
S. aureus JL-20116	1 (1)
S. aureus JL-20117	2 (1–2)
S. aureus JL-20118	2 (1–2)
S. aureus JL-20119	1 (1)
S. aureus JL-20120	2 (1–2)
S. aureus JL-20121	1 (1)
S. aureus JL-20122	2 (1–2)
S. aureus JL-20123	1 (1)
S. aureus JL-20124	1 (1)
S. aureus JL-20125	1 (1)
S. aureus JL-20126	1 (1)
S. aureus JL-20127	1 (1)
S. aureus ATCC 29213	1 (1)